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Immunohistochemical Quantification of Heparan Sulfate Proteoglycan and Collagen IV in Skeletal Muscle Capillary Basement Membranes of Patients With Diabetic Nephropathy

Hiroki Yokoyama, Poul E. Høyer, Pernille M. Hansen, Jacob van den Born, Tonny Jensen, Jo H.M. Berden, Torsten Deckert, and Charly Garbarsch

In IDDM patients, an increased permeability of the glomerular capillaries has been associated with a general loss of negatively charged heparan sulfate proteoglycans (HSPGs) within basement membranes (BMs). In the present study, we used immunohistochemical staining to quantify heparan sulfate (HS), HSPG core protein, and collagen IV in capillary basement membranes of skeletal muscle biopsies taken from 9 healthy control subjects (C) and 20 IDDM patients: 7 with normal albumin excretion rate (<30 mg/24 h) (D_0), 5 with incipient nephropathy (albumin excretion rate 30–300 mg/24 h) (D_1), and 8 with clinical nephropathy (albumin excretion rate >300 mg/24 h) (D_2). In the capillaries, staining was measured by a scanning and integrating microspectrophotometer. A significant difference in the absorbance of HS was found among the four subgroups (means ± SD): 0.477 ± 0.082 (C), 0.627 ± 0.031 (D_0), 0.542 ± 0.098 (D_1), and 0.371 ± 0.118 (D_2) (P = 0.006). Similarly, an overall significant difference in the absorbance of collagen IV was demonstrated (means ± SD): 0.836 ± 0.111 (C), 0.838 ± 0.300 (D_0), 0.970 ± 0.173 (D_1), and 0.512 ± 0.248 (D_2) (P = 0.02). No statistical difference in the absorbance of core protein was demonstrated among the groups. Within the diabetic groups, HS was inversely correlated to albuminuria (r = –0.76, P = 0.003) and albuminuria corrected for creatinine clearance (r = –0.69, P = 0.008). Because, in IDDM patients with albuminuria, alterations of the content of HS and collagen IV within the capillary BM have been demonstrated immunohistochemically, not only in the glomerular filtration barrier, but also in the skeletal muscle capillary BM, we suggest that these changes reflect universal quantitative or qualitative alterations within the capillary filtration barrier. *Diabetes* **46:1875–1880, 1997**

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AER, albumin excretion rate; BCIP, 5-bromo-4-chloro-indolyl phosphate; BM, basement membrane; BSA, bovine serum albumin; ECM, extracellular matrix; GBM, glomerular basement membrane; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; NBT, nitroblue tetrazolium; TBS, Tris-buffered saline.

According to the Steno hypothesis (1), in IDDM patients, albuminuria reflects not only severe renal disease, but also a generalized vascular dysfunction, demonstrated by increased cardiovascular morbidity and mortality (2,3). The nature of such universal alterations is unknown, and might be due to both increased capillary pressure (4) and structural alterations of the macromolecular pathway between the capillary lumen and the lymphatic capillaries (i.e., the extracellular matrix [ECM]) (5). Within the ECM, heparan sulfate proteoglycans (HSPGs) constitute a major class of proteoglycans that are found especially in basement membranes (BMs). HSPGs are formed by a central core protein to which the anionic glycosaminoglycan heparan sulfate (HS) is linked (6).

In diabetic nephropathy, renal pathobiochemical alterations are characterized by accumulation of BM-like material within the glomeruli (7) and a decreased number of anionic sites (i.e., HS or HSPG) within the glomerular basement membrane (GBM) (7–12). In rats, loss of GBM-HS induces albuminuria (13), and in humans a negative correlation between urinary albumin excretion rate (AER) and the content of HS within the GBM has been demonstrated (12). However, patients with diabetic nephropathy suffer from not only increased macromolecular permeability within the glomeruli, but also an increased extrarenal capillary permeability, as indicated by a 50% increase of the transcapillary escape rate of albumin and other plasma proteins (14,15). Therefore we hypothesized that deficiency of HS, both in glomeruli and in the vessel walls of extrarenal tissue, is the common link between albuminuria and macrovascular disease in diabetic patients with nephropathy. The aim of the present study was to evaluate possible alterations of the amount of HS in extrarenal tissue among healthy subjects and IDDM patients with and without albuminuria. We performed a quantification of HS, HSPG core protein, and collagen IV by immunohistochemistry on the BMs of skeletal muscle capillaries.

RESEARCH DESIGN AND METHODS

Subjects. Nine healthy control subjects (C) and twenty IDDM patients participated in the study. The diabetic patients were recruited from the outpatient clinic of the Steno Diabetes Center, and the control subjects were selected from the same geographic area. All IDDM patients had diabetes diagnosed before age 31 years. Diabetes duration was >10 years. All patients, except one, were on insulin treat-

TABLE 1
Clinical and laboratory data of healthy control and IDDM patients with normoalbuminuria (AER ≤30 mg/24 h [D_0]), incipient nephropathy (AER 30–300 mg/24 h [D_1]), and clinical nephropathy (AER >300 mg/24 h [D_2])

	Control subjects	D_0	D_1	D_2
n	9	7	5	8
Sex (M/F)	6/3	4/3	4/1	8/0
Age (years)	25 ± 3	36 ± 6	40 ± 10	34 ± 5
Diabetes duration (years)	—	22 ± 8	24 ± 10	21 ± 6
AER (mg/24 h)*	9 (6–17)	8 (4–22)	79 (38–143)	822 (317–5,799)
HbA_{1c} (%)	5.1 ± 0.4	8.1 ± 1.9	8.5 ± 1.5	9.5 ± 1.2
Insulin dosage (IU · kg^{-1} · 24 h^{-1})	—	0.69 ± 0.15	0.52 ± 38	0.62 ± 0.25
Systolic blood pressure (mmHg)*	120 ± 11	119 ± 11	138 ± 19	142 ± 14
Diastolic blood pressure (mmHg)*	75 ± 7	75 ± 6	85 ± 6	88 ± 5
BMI (kg/m^2)	23.2 ± 2.6	24.2 ± 1.6	25.6 ± 1.4	24 ± 1.5
Creatinine clearance (ml/min)	123 ± 60	110 ± 56†	121 ± 31	100 ± 33†
Albuminuria/creatinine clearance (μg/ml)*	0.077 (0.024–0.163)	0.064 (0.0037–0.13)†	0.41 (0.262–0.848)	6.54 (2.09–18.98)†

Data are means ± SD or median (range). *$P < 0.05$ between diabetic groups. †Values missing from one subject.

ment from the onset of diabetes, and were given 2–4 daily injections of short- and intermediate-acting insulin. No subjects were taking drugs other than insulin. The diabetic patients were divided into three groups on the basis of the median of the last three determinations of AER: group D_0 ($n = 7$) had normal AER (AER ≤30 mg/24 h), group D_1 ($n = 5$) had incipient albuminuria (AER 30–300 mg/24 h), and group D_2 ($n = 8$) had clinical nephropathy (AER >300 mg/24 h). None of the subjects studied had known nondiabetic renal or cardiac diseases, and all had urine cultures negative for bacteria. Further clinical characteristics of the study subjects are listed in Table 1. The protocol was approved by the Regional Scientific Ethical Committee, and all subjects gave informed consent.

Study design. The studies were performed in the morning. Blood pressure was measured after a 30-min rest in the horizontal position using a clinical sphygmomanometer (cuff 25 × 12 cm), and expressed as the mean of three measurements. Thereafter, subjects had morning insulin and breakfast. After a further 3-h rest, a skeletal muscle tissue specimen was taken from musculus vastus lateralis, using Bergströms biopsy needle. Xylocaine 1% was used to anesthetize the skin. The biopsies were immediately rinsed from blood and dissected free from visible fat and connective tissue. After being rinsed in saline and blotted on a wet filter paper, the tissue was divided into several samples, each rapidly frozen in isopentan and subsequently stored in liquid nitrogen at –80°C until immunohistochemical analysis was performed.

Antibodies. The immunohistochemical analyses were performed using three primary monoclonal antibodies: a mouse IgM-antibody (JM403) against HS, a mouse IgG1-antibody (JM72) against HSPG core protein, and a mouse anti-human IgG1 antibody against collagen IV. As secondary antibodies, biotinylated rabbit anti-mouse IgM antibody (cat. no. E0465; Dakopatts, Glostrup, Denmark) was used for JM403, and biotinylated rabbit anti-mouse IgG antibody (cat. no. E0354; Dakopatts) was used for JM72 as well as for mouse anti-human collagen IV antibody.

JM403 was originally raised against rat HS glycosaminoglycan, but has also been shown to react with human HS (13,16). Recently, the HS saccharide epitope responsible for the JM403 binding has been revealed. The epitope contains one or more N-unsubstituted glucosamine and D-glucuronic acid units, and is located in a region of the heparan sulfate chain composed of mixed N-sulfated and N-acetylated disaccharide units (19). Although the exact characterization of the epitope recognized by the JM72 antibody has not been revealed, it has been demonstrated that the epitope is specifically located on the human GBM-HSPG core protein and not to HS (16). According to the manufacturer (cat. no. M785; Dakopatts), the specific binding site for the collagen IV antibody has not been identified.

In the present study, the specificity of JM403 was confirmed by a considerably reduced staining intensity in sections pretreated with heparitinase.

Immunohistochemical methods. Frozen skeletal muscle tissue was cut at 6 μm in a Leitz 1720 digital cryostat (Leitz, Rockleigh, NJ) at –20°C and stored at –80°C. Four consecutive sections were separately placed on the same slide. At least two slides with sections cut from different parts of the biopsy were used for incubations. On each slide, one section each was used to detect HS side chain (Fig. 1*A*), HSPG core protein, and collagen IV. To check the specificity of the incubation procedure, one section was used as a negative control and incubated with only the secondary antibody (Fig. 1*B*). Finally, all slides from the same biopsy were given a separate code. Sections were allowed to dry at room temperature before they were fixed in 100% acetone. Unless otherwise noted, all dilutions were performed in Tris-buffered saline (TBS) (0.15 mol/l NaCl in 0.05 mol/l Tris buffer; pH 7.6). All reactions were performed at room temperature.

To block nonspecific background, sections were incubated for 30 min with normal rabbit serum (cat. no. X0902; Dakopatts) diluted 1:10 in TBS with 1% bovine serum albumin (BSA). Excess serum was wiped off. Primary antibodies diluted in TBS with 1% BSA were added. In initial experiments, optimal antibody dilutions were estimated as values restricted to the steep part of the absorbance versus dilution curve: JM403 1:16000, JM72 1:8000, and anti-human collagen IV antibody

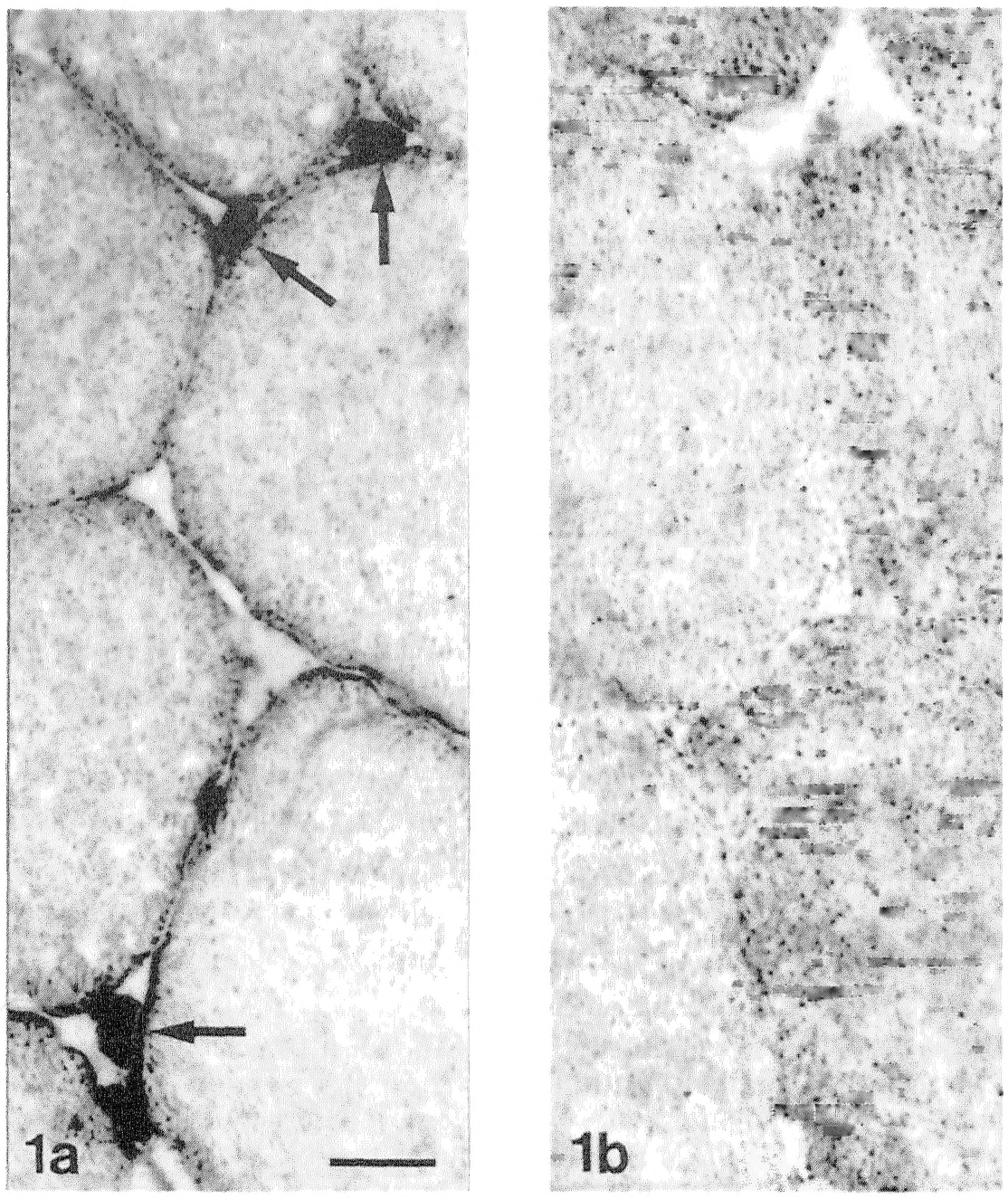
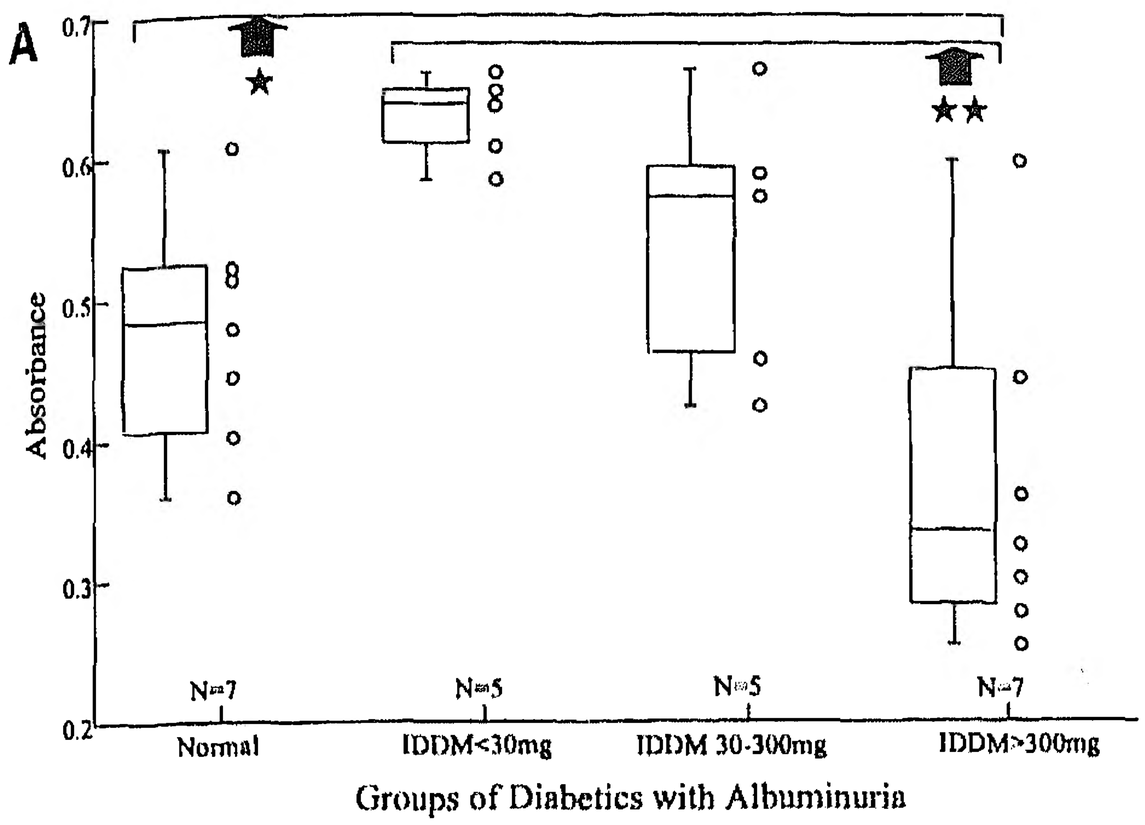
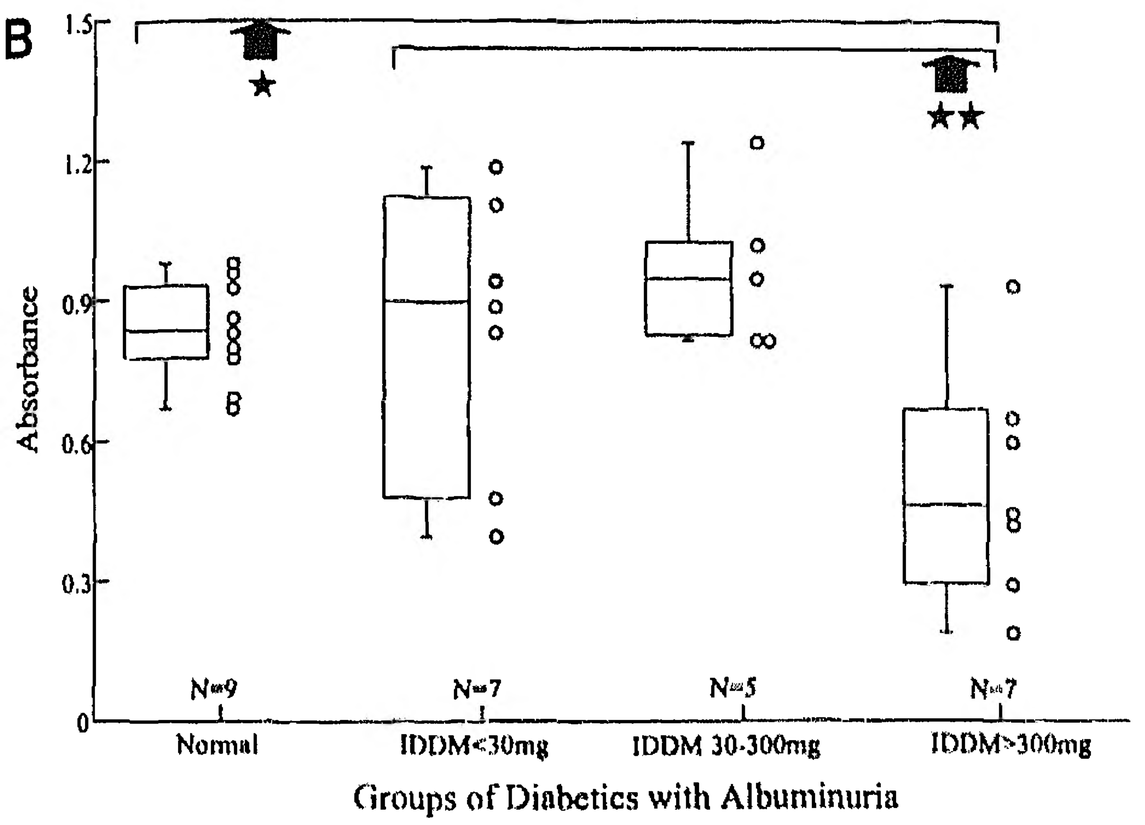


FIG. 1. *A*: cross section of adjacent skeletal muscle fibers. The microphotograph depicts the position of the JM403 antibody against HSPG side chains in the muscle capillary BM. The final reaction product of BCIP/NBT does not demonstrate the BM per se, but illustrates the BM via the alkaline phosphatase reaction of the secondary antibody (magnification ×500). Bar 20 μm = 1 cm. *B*: microphotograph illustrating the negative control section to which the primary antibody is not applied (magnification ×500).





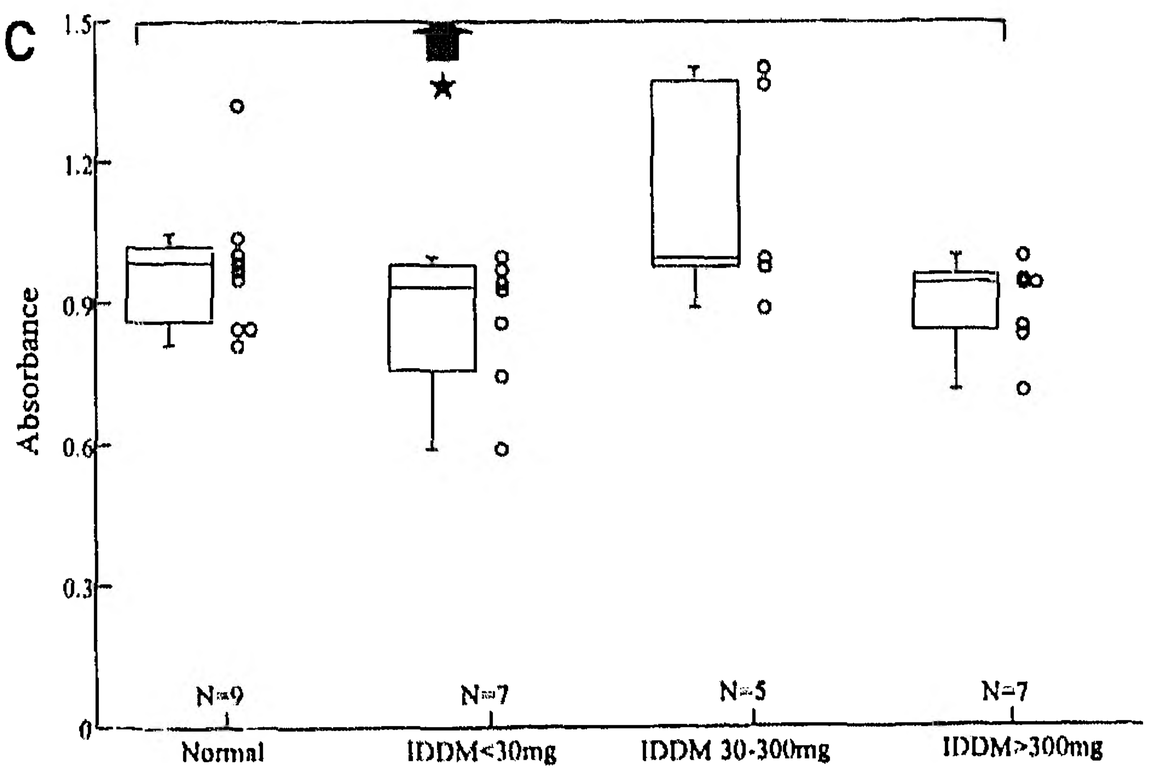


FIG. 2. *A*: absorbance of HS. ★, $P < 0.006$ among all groups; ★★, $P = 0.01$ among diabetic groups. *B*: absorbance of collagen IV. ★, $P = 0.02$ among all groups; ★★, $P = 0.03$ among diabetic groups. *C*: absorbance of HSPG. ★, $P = 0.12$ among all groups and diabetic groups. Within subgroups, the box-and-whisker plot estimates the median, upper, and lower quartiles and the range; points estimate the mean value in individual subjects.

1:48000. The sections were incubated overnight at 4°C. After being washed in three changes (5 min each) of TBS, secondary antibodies diluted 1:250 (biotinylated anti-mouse IgM antibody) and 1:200 (biotinylated rabbit anti-mouse IgG antibody) were added; the sections were then incubated for 30 min. Another three changes (5 min each) in TBS were performed, after which the sections were incubated for 30 min with alkaline phosphatase-conjugated avidin according to the manufacturer's prescription (K0376; Dakopatts). This was followed by three changes (5 min each) in TBS. Finally, alkaline phosphatase activity was revealed by development for 20 min in 5-bromo-4-chloro-indolyl phosphate/nitroblue tetrazolium medium containing 1 mmol/l levamisole (Sigma, St. Louis, MO). The medium was prepared by mixing the following solutions: 10 ml predetection buffer (100 mmol/l Tris buffer [pH 8.8], 5 mmol/l $MgCl_2$, and 100 mmol/l NaCl), 20 µl 5-bromo-4-chloro-indolyl phosphate solution (BCIP; 10 mg BCIP [Sigma, St. Louis, MO] in 200 µl *N,N*-dimethylformamide) and 140 µl nitroblue tetrazolium solution (NBT; 15 mg NBT [Sigma, St. Louis, MO] in 200 µl *N,N*-dimethylformamide).

All procedures for collagen IV, HS, and HSPG core protein for all subjects were performed in one setting.

Quantitative procedure. A minimum of 40 muscle capillaries cut in cross section and located between at least three adjacent muscle fibers (e.g., Fig. 1*A*) were randomly selected from at least two sections on slides with the same code. If it was impossible to identify 40 capillaries for quantification, the specimen was excluded. A randomly selected area of the stained BM in each of the capillaries was scanned with a Vickers M85A scanning and integrating microspectrophotometer (Vickers, York, U.K.) with the following machine settings: ×100 objective (scanning area 3.1 μm^2), light at the isobestic wavelength (585 nm) of NBT formazans, and a scanning spot diameter of 0.2 µm. Within each area, the individual spot readings were automatically integrated by the instrument. The mean integrated absorbance over all areas was then calculated by reference to a standard calibration graph constructed by measuring a series of neutral density filters with known absorbance values. Because this procedure was performed on each experimental day, any day-to-day variation in the sensitivity of the microspectrophotometer was eliminated. Moreover, measurements of the neutral density filters showed that the instrument was linear within the range of absorbance values recorded in the capillaries. Finally, the mean formazan deposition measured in the absence of the primary antibody was subtracted from that measured in the presence of antibody to give a measure (± SD) of the specific antibody binding alone.

Metabolic control and renal function. HbA_{1c} was measured by high-performance liquid chromatography (Bio Rad DIAMAT, Richmond, CA; normal range 4.3–6.2%). Blood glucose was measured by means of Hypocount B (Hypoguard LD, Woodbridge, Suffolk, U.K.). Serum and urinary creatinine was measured by a kinetic Jaffe's method (interassay coefficient of variation <5%). Urinary albumin concentration was determined using an enzyme-linked immunosorbent assay method (intra- and interassay coefficient of variation <6%) (17). In diabetic nephropathy, the glomerular filtration rate and creatinine clearance show a strong inverse correlation with the percentage of occluded and globally sclerosed glomeruli (18). We therefore corrected the albuminuria (mg/24 h) for the creatinine clearance (ml/min), yielding a ratio expressed in µg/ml (Table 1). This ratio of albuminuria reflects the albumin filtration in still-functioning glomeruli (12).

Statistical analyses. The slides were decoded at the end of the experiment. Data were processed using the StatGraphics statistical software package (STSC, Rockville, MD) and Unistat for Windows (Unistat, London, U.K.). Results are expressed as means ± SD, except for results from AER. AER was not normally distributed, and the results are therefore given as median and range. Differences between groups were assessed by unpaired Kruskal-Wallis tests. Correlations were expressed by Spearman's correlation coefficients (r). P values <0.05 were accepted as significant.

RESULTS

Clinical characteristics of the study groups are given in Table 1. The diabetic groups were comparable regarding age, diabetic duration, daily insulin dosage, and BMI. Among diabetic patients, systolic and diastolic blood pressure was significantly higher in patients with albuminuria. A similar increase was observed for HbA_{1c}, although it was not significant.

Because of the exclusion criteria related to the quantitative analysis, HS, HSPG core protein, and collagen IV were quantified in an unequal number of subjects. HS was quantified in 24 subjects. An overall significant difference was found among the four subgroups ($P < 0.006$) and among the diabetic groups ($P = 0.01$; Fig. 2*A*). This was mainly due to an increase in HS absorbance within the normoalbuminuric group of

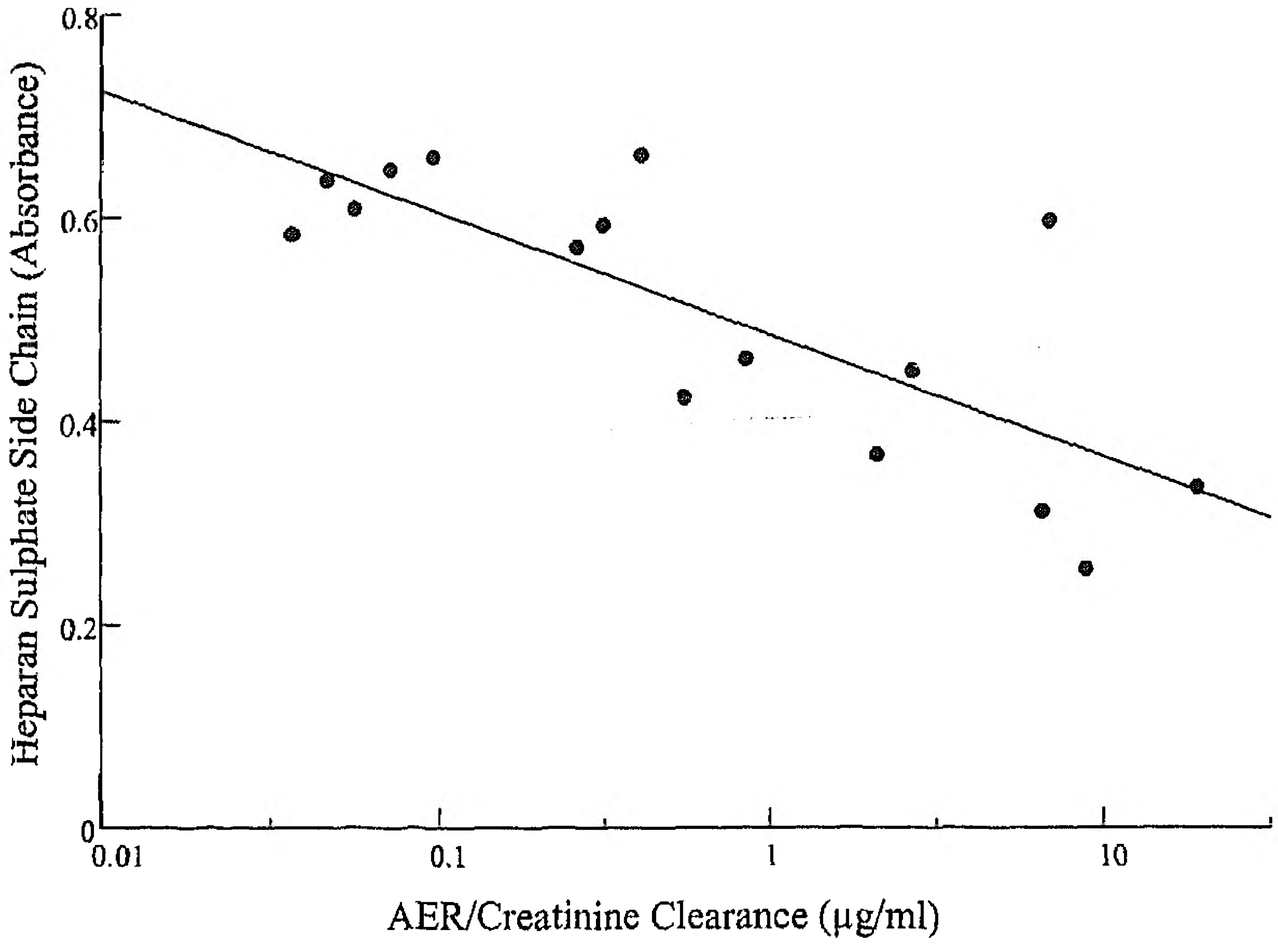


FIG. 3. HS absorbance versus albuminuria/creatinine clearance (µg/ml) in patients with IDDM (n = 16, creatinine clearance is missing in 1 patient); r = –0.69, P = 0.008.

IDDM patients and a reduction of HS absorbance in the clinical nephropathy patients: 0.477 ± 0.082 (C), 0.627 ± 0.031 (D_0), 0.542 ± 0.098 (D_1), and 0.371 ± 0.118 (D_2). Within the diabetic patients, a significant inverse correlation between HS absorbance and albuminuria (r = –0.76, P = 0.003) or albuminuria corrected for creatinine clearance (r = –0.69, P = 0.008; Fig. 3) was observed.

Collagen IV was quantified in 28 subjects. An overall significant difference in absorbance was found among the four subgroups (P = 0.02) and among the three diabetic groups (P = 0.03; Fig. 2*B*). This was mainly due to a decrease of collagen IV within the group of patients having clinical nephropathy: 0.836 ± 0.111 (C), 0.838 ± 0.300 (D_0), 0.970 ± 0.173 (D_1), and 0.512 ± 0.248 (D_2). Within the diabetic group, collagen IV absorbance revealed a significant inverse correlation with albuminuria corrected for creatinine clearance (r = –0.57, P = 0.02). The coefficient of variation was 3.5, 2.2, and 2.8%, respectively, when three sections stained for collagen IV were measured on each of 10 days.

HSPG core protein was quantified in 28 subjects. Among the groups no significant difference in absorbance was revealed: 0.985 ± 0.150 (C), 0.868 ± 0.146 (D_0), 1.126 ± 0.24 (D_1), and 0.894 ± 0.097 (D_2) (P = 0.12; Fig. 2*C*).

No correlation between HS absorbance and HbA_{1c}, postprandial blood glucose, or diabetes duration was demonstrated in the entire population or within the diabetic groups. Also, the absorbance of collagen IV and HSPG core protein was unrelated to HbA_{1c}. Furthermore, if the diabetic subjects enrolled for the measurements of HS, collagen IV, or HSPG core protein absorbance were stratified into two groups defined by a HbA_{1c} level above or below the median value, no significant difference in absorbance was demonstrated between groups.

DISCUSSION

Previously, a comparable decrease of GBM-HS or HSPG core protein has been demonstrated in diabetic kidney disease, although the results have been restricted by the use of semiquantitative analysis (7,9,10,12). In the present study, a reproducible quantitative immunohistochemical analysis was used to estimate the concentration of HS, HSPG core protein, and collagen IV within capillary BMs of skeletal muscle biopsies. The present data document for the first time an extrarenal reduction of capillary BM-HS in patients with diabetic nephropathy.

Such an alteration of BM-HS absorbance in IDDM patients may be explained by altered antibody-epitope interaction. Recently, we showed that the JM403 binding to HS is dependent on the presence of an N-unsubstituted glucosamine unit in HS, and that the JM403 epitope expression in HS preparations from various sources is inversely correlated with HS sulfation (19). The HS chains are modified posttranscriptionally by N-substitution of the glucosamine units, facilitated by the key enzyme glucosaminyl N-deacetylase/N-sulphotransferase. This enzyme has been found to be decreased in both diabetic animals (20,21) and diabetic patients (22). Thus, in the present study, the increase of HS absorbance in the D_0 group might be related to an undersulfation of HS. Consequently, our finding of decreased HS absorbance in patients with albuminuria might be explained by oversulfation of HS; that is, a decrease in the number of N-unsubstituted glucosamine units of HS or a decreased content of HS in the BM of muscle capillaries. Along the same line, our observation of decreased absorbance of HS in the D_2 group might be due to an absolute decrease of HS within muscle capillary BM, as was previously demonstrated in the GBM of diabetic patients with albuminuria (11). We therefore suggest that the first change in HSPG in diabetes is probably an undersulfation of HS (the D_0 group), followed by an absolute decrease of HS (the D_1 and D_2 groups), as has been discussed previously (23). An absolute reduction of BM-HS could be genetically determined. Thus an altered gene transcription or posttranscriptional modification of the first domain within the HSPG2-gene coding for the amino acid sequence at the protein HS-attachment sites (24) could be responsible for the observed "loss" of HS. Such a qualitative alteration within the HSPG core protein might not have been detected in the present quantification.

Theoretically, the reduced quantity of HS could be due to masking of epitopes, either by nonenzymatic glycation of the

primary amino group on HS recognized by JM403, or by increasing amounts of other BM proteins, such as collagens, laminin, or fibronectin (7,9), perhaps induced by transforming growth factor-β overexpression (25). Thus it has been interpreted that structural changes in the GBM prevent the normal organization of HSPG, making it inaccessible to specific antibodies (26), and thereby blurring the use of immunohistochemistry to draw conclusions about quantitative changes in macromolecular compositions. In the present study, the influence of nonenzymatic glycation seems to be negligible, since HS absorbance did not correlate with metabolic regulation. Also, a relationship between collagen IV and a reduction of HS seems unlikely, given that a concomitant decrease of both molecules was found in diabetic patients with nephropathy. This is in agreement with the findings of Tamsma et al. (12), who studied the GBM of IDDM patients with advanced nephropathy, but disagrees with findings from other studies in which increasing amounts of GBM-collagen IV in diabetic patients with albuminuria were reported (7,9,27). Conflicting results might be due to the inclusion of patients with different degrees of albuminuria, since it has been demonstrated that collagen IV expression is increased in the early stage of diabetic nephropathy and decreased in more advanced stages (28,29). Further, different results might be obtained due to the use of anti–collagen IV antibodies with different specificities (directed against either α_1/α_2 or α_2/α_1). We used a commercial monoclonal anti–collagen IV antibody in which the specific binding sites have not been identified.

In accordance with two former studies, we demonstrated that the content of BM-HS in muscle capillaries is inversely correlated to the degree of albuminuria (9,12). Because an increased glomerular leakiness of albumin in diabetic patients has been related to an increased systemic transcapillary albumin escape rate (14), we interpret our data as supporting the hypothesis of a functional relationship between a decreased content of BM-HS and a general increased transcapillary escape rate of albumin (1), although in the present study the latter could not be measured. In addition, our data demonstrated an inverse correlation between collagen IV absorbance and albuminuria corrected for creatinine clearance, thereby indicating that, besides HS, changes in collagen IV might also contribute to an enhanced diabetes-related ECM permeability.

Acknowledging that capillary wall transport involves mechanisms other than charge-electrostatic interactions in BMs (30), the major impact of the present study is that, using the same primary antibodies, a similar reduction in BM-HS of muscle capillaries and GBM-HS (12) is observed in IDDM patients with nephropathy. Accordingly, we suggest that albuminuria in insulin-dependent diabetic patients reflects universal quantitative or qualitative alterations of the capillary filtration barrier.

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